Troubleshooting the Western blot procedure

Problem: Chemiluminescent or chromogenic signal weak or not visible

Possible cause	Remedy
Poor isolation of tagged protein	Use a different cell lysis procedure
Antibody too dilute	Double the concentration of the Tag-specific Antibody (and/or the Secondary Antibody)
Too little protein on the gel	Add more protein to gel.
Poor transfer of proteins from gel to membrane *(Note: Check efficiency of transfer by silver staining the remaining gel. There should be minimal protein left in the gel.)*	1) Increase the electrical current and/or the transfer time for the blot. 2) Be sure there are no air bubbles between the membrane and gel during transfer.
Wrong type membrane	For maximum signal, use PVDF membranes for transfer.
Antibody incubation too short	Incubate the Tag-specific Antibody (and/or the Secondary Antibody) with the membrane blot for a longer time.
Signal development time too short	Double the development time.
Wash time too long or too stringent	1) Shorten the washing time. 2) Omit Tween 20 from the Wash Buffer.
Enzyme on antibody conjugate inactivated by preservative *(Note: To check enzyme activity, dot increasing dilutions of the antibody conjugate onto a membrane and perform the visualization reaction directly on the dilutions.)*	If you use POD-conjugated antibodies, do not use sodium azide in any Western blot reagent. If you use AP-conjugated antibodies, do not use a phosphate buffer in the washes following the incubation with Secondary Antibody; substitute a Tris buffer.
Substrate inactive *(Note: Check reagent on dot blot containing dilutions of antibody conjugate with established enzyme activity.)*	Make fresh dilution of substrate or start with a different stock of substrate.
Epitope tag sequence is not detectable due to:	
• Proteolytic cleavage	Include protease inhibitors in lysis buffer.
• Low level of expression	Use alternative expression system or optimize your expression system. Insert multiple tag sequences into target protein to increase avidity of antibody reaction.
• Premature translation termination resulting in loss of C-terminal tag sequence	Use alternative insertion site within the target gene for the epitope tag sequence.

Problem: High background, additional bands on blot

Possible cause	Remedy
Antibody too concentrated	Decrease concentration of Tag-specific Antibody (and/or Secondary Antibody) by half.
Wash time too short	Increase washing times.
Incubation of membrane with substrate too long	Leave blot membrane in substrate for a shorter time.
Wrong type membrane	For minimum background, use PVDF membranes for transfer.
Blocking Reagent too dilute	Use nonfat dry milk (5% w/v) dissolved in Reagent Diluent as Blocking Reagent. (*Caution: High concentrations of nonfat dry milk may reduce specific signal as well as background*.)
Contaminated reagents or equipment	1) Use clean equipment, freshly prepared buffers, and new membranes. 2) Always avoid touching membranes with bare hands; use gloves and forceps.
Secondary antibody binds untagged proteins	Use an $F(ab')_2$ fragment of a secondary antibody, rather than an intact IgG.
Heavy and light chains of primary antibody visible on blot membrane (Indirect detection procedures only)	Use direct detection with peroxidase-conjugated monoclonal antibody to visualize tagged proteins.
Signal development time too long	Reduce development time by half.